11-ARACHIDONATE-LIPOXYGENASE MUTANTS

The present invention relates to a method for producing a plant lipoxygenase with modified positional specificity and to the lipoxygenase obtained by said method and to the use thereof for the hydroperoxylation of arachidonic acid to carbon atom 11.

Lipoxygenases (LOXs, linolic acid: oxygen oxidoreductase; EC.1.13.11.12; LOXs) are widely distributed in the plant and animal kingdom (Siedow, J.N. (1991) Ann. Rev. Plant Physiol. Plant Mol. Biol. 42, 145-188; Yamamoto, S. (1992) Biochim. Biophys. Acta 1128, 117-131). These enzymes constitute a family of iron-containing dioxygenases that catalyze a region- (or position-) and stereo-selective oxygenation of polyenoic fatty acids to hydroperoxy derivatives (Rosahl, S. (1996) Z. Naturforsch. 51c, 123-138). In mammals, LOXs are classified according to their specificity for specific positions during arachidonic acid oxygenation (Yamamoto, S. (1992) Biochim. Biophys. Acta 1128, 117-131; Schewe, T., Rapaport, S.M. & Kühn, H. (1986) Adv. Enzymol. Mol. Biol 58, 191-272). 15-, 12-, 8- and 5-LOXs have so far been isolated here. LOXs which effect the insertion of oxygen at positions 9 and 11, respectively, on the carbon skeleton of arachidonic acid have not been known yet (Yamamoto, S. (1992) Biochim. Biophys. Acta 1128, 117-131). Since arachidonic acid either is not present in higher plants or is only present in small amounts as a constituent of storage lipids, plant LOXs are classified as 9- and 13-LOXs. This nomenclature is derived from the position at which oxygenation takes place in linolic acid (LA) (Gardner, H.W. (1991) Biochim. Biophys. Acta 1084, 221-239). Recently, a more comprehensive classification of plant LOXs has been proposed based on a comparison of the primary structures (Shibata, D. & Axelrod, B. (1995) J. Lipid Mediators Cell Signal. 12, 213-228). The specificity of a LOX for a specific position is the result of two catalytic partial reactions:

(i)
Regio- and stereospecific hydrogen removal; in fatty acids containing several double bonds (such as linolenic acid, arachidonic acid, or eicosapentaenoic acid), hydrogen removal is possible at various positions.

(ii)
Regio- and stereospecific oxygen insertion (oxygen can be inserted at various positions (either at the +2 or –2 position)) (cf. Fig. 1). Thus, a fatty acid containing three double-allylic methylenes, such as arachidonic acid, can be oxygenated by a LOX to six regioisomeric hydroperoxy derivatives (HPETEs), namely 15- and 11-HPETE (these originate from the removal of hydrogen at position C-13), 12- and 8-HPETE (these originate from hydrogen removal at position C-10) and 9- and 5-HPETE (these originate from hydrogen removal at position C-7). Experiments on mammalian 12- and 15-LOXs indicated that the position of hydrogen removal can be altered when critical amino acids are changed by directed mutagenesis (Borngräber, S., Kuban, R. J., Anton, M. & Kühn, H. (1996) J. Mol. Biol. 264, 1145-1153; Sloane, D.L., Leung, R., Craik, C. S. & Sigal, E (1991) Nature 354, 149-152). Attempts to alter the LOX reactivity from a +2 to a –2 rearrangement or vice versa (e.g., conversion of a linoleate 13-LOX to a 9-LOX) by directed mutagenesis have recently been successful (Hornung, E., Walther, M., Kühn, H. & Feussner, I. (1999) Proc. Natl. Acad. Sci. USA 96, 4192-4197).

It has been the object of the present invention to indicate a method for providing a LOX with a desired C-11 positional specificity in arachidonic acid.

This problem is solved according to the invention by a method in which at least one amino acid is exchanged in a wild type LOX, preferably of potato tuber.

Figure 1 shows the specificity of a LOX reaction with substrates containing two allylic methylenes.

Figure 2 shows the HPLC analysis of hydroxy fatty acids obtained with the help of wild type LOX of potato tubers and the V576F mutant of arachidonic acid after reduction of hydroperoxy fatty acids with sodium borohydride.

Sub A3 Figure 3 shows the sequence of wild type LOX of potato tubers. The mutagenized amino acid position is underlined. Primers 1 und 2 as used are also shown.

In a preferred embodiment, the amino acids are exchanged in the region of the amino acid position 570 to 581 of potato tuber LOX. The above-indicated amino acid positions refer to the sequence under the access number S73865 in the EMBL data base or the sequence according to Fig. 3. The positions in LOXs of other plant species, which correspond to the amino acid positions 593 to 602 of *Cucumis sativus* lipoxygenase, can easily be determined by sequence comparisons between sequence X92890 and the further protein sequences, e.g., of soybean, potato, arabidopsis, tobacco or barley. The following Table 1 shows the result of an amino acid comparison between the cucumber-derived enzyme and the corresponding positions in the enzymes of other plants. The first group (15-LOX) shows a comparison between LOXs which at position 15 introduce a hydroperoxy group into an arachidonic acid molecule, while the second group (5-LOX) shows a comparison between sequences which introduce a hydroperoxy group at position 5.

Table 1

Comparison of amino acid residues involved in the specificity of a plant LOX for a specific position (15 or 5).

ENZYME	Access No.	Position of amino acid residue	Amino acid residue
15-LOX			
Cucumber lipid body LOX	X92890	596/597	Thr/His
Soybean seed LOX-1	P08170	556/557	Thr/Phe
Potato LOX-H1	X96405	614/615	Ser/Phe
Arabidopsis LOX-2	P38418	611/612	Cys/Phe
5-LOX			
Potato LOX	S73865	575/576	Thr/Val
Tobacco elicitor-induced LOX	X84040	580/581	Thr/Val
Barley grain LOX-A	L35931	574/575	Thr/Val

The sequence motif at position 570 to 581 is GVLESTVFPSK (sequence according to S73865).

In a particularly preferred embodiment the exchange takes place at position 576 of sequence S73865. At position 576, there is a Val residue in the wild type. The residue at position 576 is here replaced by a Phe residue. The exchange in the region of the amino acid position 570 to 581 has the effect that the potato tuber 5-LOX is converted to an arachidonic acid 11-LOX. In the following this mutant will also be designated as V576F. The wild type sequence is shown as Fig. 3. Position 576 is marked.

Preferably, the amino acids are exchanged in the wild type with the help of directed mutagenesis, as is sufficiently known in the prior art.

The present invention further relates to LOX mutants which are obtainable according to the above-described methods. The LOXs according to the invention can be produced with the help of the methods known from the prior art, for example directed mutagenesis, and subsequent protein expression. In particular mutants which after incubation with arachidonic acid yield at least 40%, preferably 50%, of the derivative perhydroxylated to position 11 are considered to be inventive.

The present invention further relates to nucleic acids which code for the LOXs according to the invention. Starting from the wild type sequences available in the prior art, the sequences according to the invention can be produced by directed mutagenesis.

Furthermore, the present invention relates to vectors into which the nucleic acids according to the invention are introduced for the purpose of cloning and expression. Corresponding cloning and expression vectors are sufficiently known to the skilled artisan from the prior art (cf. Maniatis et al. Molecular Cloning, A Laboratory Manual (1989), Cold Spring Hator Laboratory Press).

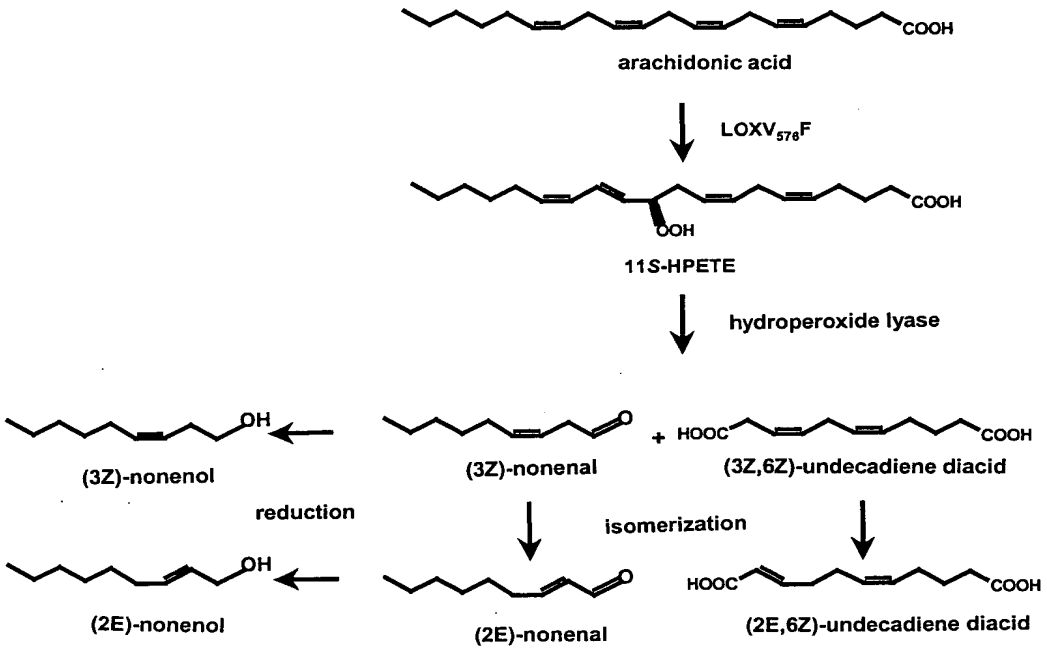
The present invention further relates to a cell into which the nucleic acid according to the invention or the vector according to the invention is introduced. After introduction of the nucleic acid or the vector, the cell is then capable of expressing a LOX for the first time or at a large scale. The fatty acid pattern of a cell can thereby be changed in a targeted way, with the result that the phenotype of the cell can be altered in different respects. This includes, inter alia, a different composition of the cell membrane.

Finally, new plants or plant parts can be regenerated from the above-mentioned cells by *in vitro* culturing methods. For the production of such transgenic plants the known transformation system can e.g. be used on the basis of *agrobacteria* and Ti plasmid derivatives.

The LOXs according to the invention make it possible to produce for the first time new arachidonic acid derivatives at a large scale. To this end, arachidonic acid is incubated as a substrate with the LOXs according to the invention under appropriate

conditions. Hydroperoxylation of the arachidonic acid is then effected, preferably at position 11.

Particularly preferred is an arachidonic acid derivative which contains a hydroperoxy group at position 11. The derivative can then easily be converted into the hydroxy derivative. The thus available 11S-HPETE can be used for producing the alcohols, aldehydes and dicarbonic acids shown below. The enzyme hydroperoxide lyase is e.g. contained in extracts of cucumber seedlings. 2E- and 3Z-nonenal and their alcohols are important flavorings in foodstuff (e.g. cucumbers).



Such an arachidonic acid derivative has so far not been available because a LOX of an appropriate positional specificity has been missing.

The further examples serve to explain the invention.

1. Preparation of the mutant V576F

Materials:

The chemicals used were obtained from the following sources: standards of chiral and racemic hydroxy fatty acids were obtained from Chayman Chem (Ann Arbor, Mi, USA). Methanol, hexane, 2-propanol (all HPLC grade) were obtained from Baker (Griesheim, Germany). Restriction enzymes were purchased from New England BioLabs (Schwalbach, Germany).

Directed mutagenesis and protein expression:

For bacterial expression of wild type LOX and LOX mutant and for directed mutagenesis, use was made of the plasmid pet3b (Novagen, Germany) which contained the cDNA of the potato tuber LOX as insert (pET-LOX1; cf. Geerts, A., Feltkamp, D., Rosahl, S. (1994) Expression of lipoxygenase in wounded tubers of *solanum tuberosum* L. Plant Physiol. 105: 269-277). Mutagenesis was carried out by using the QuikChange Mutagenesis Kit from Stratagene (Heidelberg, Germany). Oligonucleotides containing the appropriate base exchanges were purchased from MWG-Biotech (Ebersberg, Germany). To analyze the mutation, an additional conservative base exchange was introduced to construct a new restriction cleavage site. In addition, the mutation was sequenced and at least five different bacterial clones were expressed and used for analyzing the enzymatic characteristics. Expression of pET-LOX1 und its mutant was performed as described by Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436. Cells from 1 liter cultures were resuspended in 5-7 ml lysis buffer and disrupted by using a sonifier tip with pulses each of 30 seconds, and cellular debris was pelleted.

Activity assay and sample pr paration:

For product analysis, 0.9 ml of cell lysates was incubated with 0.9 mM arachidonic acid (final concentration) in 100 mM Tris buffer, pH 7.5, for 30 minutes at room temperature. Reaction was stopped by the addition of sodium borohydride to convert the hydroperoxy fatty acids formed to the corresponding hydroxy compounds. The samples were acidified to pH 3 and the lipids were extracted (cf. Bligh, E.G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917). The lower chloroform phase was recovered and the solvent was evaporated. The remaining lipid was dissolved with 0.1 ml methanol, and aliquots were subjected to HPLC analysis.

Analysis:

HPLC analysis was carried out on a Hewlett Packard 1100 HPLC system coupled to a diode detector. RP-HPLC of the free fatty acid derivatives was carried out on a Nucleosil C-18 column (Macherey-Nagel, 250 x 4 mm, 5µm particle size) with a solvent system of methanol/water/acetic acid (85/15/0.1; v/v/v) and at a flow rate of 1 ml/min. Absorption at 234 nm (absorption of the conjugated diene system of the hydroxy fatty acids) and at 210 nm (polyenoic fatty acids) were recorded accordingly. Straight-phase HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (HP, Waldbronn, Germany; 250 x 4.6 mm, 5 µm particle size) with a solvent system of n-hexane/2-propanol/acetic acid (100/2/0.1, v/v/v) at a flow rate of 1ml/min. The enantiomer composition of the hydroxy fatty acids was analyzed by chiral-phase HPLC on a Chiralcel OD column (Daicel Chem. Industries, distributed by Baker Chem., Deventer, Netherlands; 250 x 4.6 mm, 5µm particle size) with a solvent system of hexane/2-propanol/acetic acid (100/5/0.1, v/v/v) at a flow rate of 1 ml/min. (Cf. Feussner, I., Balkenhohl, T.J., Porzel, A., Kühn, H.& Wasternack, C. (1997) J. Biol. Chem. 272, 21635-21641).

2. Preparation of the LOX-V576F mutant:

The reagents and methods employed for preparing said mutant were substantially as already described above. A few modifications of the above-mentioned methods which were specifically adapted to the preparation of the V576F mutant are now explained.

Directed mutagenesis and protein expression:

The starting cDNA and the mutagenesis kit were as described above. For analysis of the mutation further conservative base exchanges were carried out for producing a new restriction cleavage site for BsTBL. The following primers were used for producing the mutation V576F: GCT GGT GGG GTT CTT GAG AGT ACA TTC TTT CCT TCG AAA TTT GCC ATG GAA ATG TCA GCT G (coding strand) and CAG CGT ACA TTT GCA TGG CAA ATT TCG AAG GAA AGA ATG TAC TCT CAA GAA CCC CAC CAG C (complementary strand). Furthermore, the mutant was sequenced and 5 different bacterial colonies were expressed and used for enzymatic studies. The expression of pET-LOX1 was carried out as described above. The further preparation was carried out as already indicated above. Analysis of the produced fatty acid derivative (containing a hydroperoxy group at position 11) was carried out as indicated above. The result of the SP-HPLC analysis for converting arachidonic acid with V576F is shown in Fig. 2. The following Table 2 shows a comparison of the specificity of the wild type (wtLOX) with the mutant ($LOXV_{576}F$).

Table 2

Comparison of the product specificity of wtLOX and LOXV_{576}F with arachidonic acid

Enzyme	(15S,13*E*,11Z,8Z,5*Z*)-20:4	(12S, 14Z, 10*E*,8Z,5*Z*)-20:4	(11S,14Z,12*E*,8Z, 5*Z*)-20:4	(9*S*,14Z,11Z,7*E*, 5*Z*)-20:4	(8*S*,14Z,11Z9*E*, 5*Z*)-20:4	(5*S*, 14Z, 11Z,8Z6*E*)-20:4
wtLOX	5 %	6 %	23 %	3 %	21 %	42 %
LOXV_{576}F	9 %	4 %	50 %	3 %	23 %	11 %

3. D scription of the figures

Figure 1 shows that the positional specificity of the LOX reaction depends on the site of hydrogen cleavage and the orientation of the radical. The [+2] radical arrangement indicates that oxygen is inserted at the second carbon atom in the direction of the methyl terminus of the substrate counted from the site of hydrogen removal. [-2] indicates the inverse orientation of the radical arrangement.

Figure 2 shows HPLC analysis of fatty acids with the mutant V576F. Equal amounts of LOX protein were incubated with 0.9 mM arachidonic acid at room temperature for 30 minutes. After reduction of lipids with sodium borohydride, the reaction mixture was acidified to pH 3, and the lipids were extracted. Oxygenated fatty acid derivatives were isolated by RP-HPLC, and the individual positional isomers were analyzed by SP-HPLC. Ratios of S and R were determined by HPLC (insets).

Figure 3 shows the amino acid sequence of the wild type lipoxygenase of potato tubers. The mutated Val576 is underlined.

Abbreviations used:

CP-HPLC	for	chiral-phase HPLC;
RP-HPLC	for	reverse-phase HPLC;
SP-HPLC	for	straight-phase HPLC;
HPETE	for	hydroperoxy arachidonic acid;
LOX	for	lipoxygenase;
HETE	for	hydroxy arachidonic acid